CLEAN VERSION OF AMENDED SPECIFICATION PARAGRAPHS

MURINE 4-1BB GENE (as amended)
Applicant: Byoung S. Kwon
Serial No.: 08/012,269

Clean Version of Page 8, Paragraph 2

Figure 17 shows a comparison of the 4-1BBP amino acid sequence (SEQ ID NO:3) with the amino acid sequence in sina (SEQ ID NO:4) Drosophila and DG17 (SEQ ID NO:5) of Dictyostelium.

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The amino-terminal sequence of the purified 4-1BBPs was determined. The sequence was Val-Gln-Asn-Ser-X-Asp (SEQ ID NO:6). The amino acid sequence at positions 1, 2, 3, 4 and 6 was identical to that of the mature 4-1BBP predicted from the cDNA sequence. Amino acid at position 5 which is supposed to be Cys was not determined. These results indicate that the deduced amino acid sequence and assignment of signal sequence are correct. When the potential transmembrane domain was removed from the complete 4-1BB molecule, the protein was secreted. These results suggested that 4-1BBP was likely to be associated with the cellular membrane as predicted by the primary structure.

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Antibody Preparation. An oligopeptide representing amino acids 105-115 of the deduced 4-1BBP sequence was synthesized (Applied Biosystems). The sequence was NH_2-CRPGQELTKSGY-COOH (SEQ ID NO:7). A tyrosine residue at the C-terminus of the peptide was added for possible radioactive labeling with [^{125}I]. The peptide was conjugated to keyhole limpet hemocyanin (KLH) with a heterobifunctional cross linker, m-maleimidobenzoyl-n-hydroxysuccinimide ester **(88, 107)**.

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This region forms the pattern of C-X_2-C-X_9-H-X_3C-X-C (SEQ ID NO:8); and the cysteines and histidine are conserved in a similar space in 4-1BB, sina, and DG17 proteins. Ten of 24 amino acids between the 4-1BB and sina proteins are identical. Between 4-1BB and DG17 proteins, 11 of 24 amino acids are identical, and 3 of 24 are conservative substitutions. The conserved pattern suggests that these amino acids are functionally important.

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4-1BB contains other interesting features in its cytoplasmic domain. Those include 1) two runs of acidic amino acids; 2) a potential $p56^{lck}$ binding site; 3) five consecutive glycines at the carboxyl terminus; and 4) four potential phosphorylation sites - 1 tyrosine, 2 threonine, and 1 serine. It is especially interesting that 4-1BB contains a potential $p56^{lck}$ binding site, -C-R-C-P- (SEQ ID NO:9). The consensus sequence of $p56^{lck}$ binding site is -C-X-C-P- (SEQ ID NO:10) in the CD4 and CD8 molecules **(93)**.

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To construct a plasmid that expresses extracellular portion of 4-1BB, the putative extracellular domain of 4-1BB cDNA **(89)** was amplified by polymerase chain reaction (PCR) **(99)**. An XhoI site was created at the 5' end of the forward primer and a stop codon, (TAA), and an EcoRI site were created in the reverse primer. The PCR product was digested with XhoI and EcoRI and the -0.6 kb fragment was purified. The XhoI-EcoRI fragment (4-1BBS) was inserted into the PEV-55 vectors **(53)**, generating PEV-55-4-1BBS. The sequence of the forward primer (SEQ ID NO:11) was 5' -ACCTCGAGGTCCTGTGCATGT-GACA-3' and that of the reverse primer (SEQ ID NO:12) was 5' -ATGAATTCTTACTGCAGG-AGTGCCC-3'.

CLEAN VERSION OF AMENDED SPECIFICATION PARAGRAPHS

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This region forms the pattern of C-X_2-C-X_9-C-X_3-H-X_3-C-X-C (SEQ ID NO:8); and the cysteines and histidine are conserved in a similar space in 4-1BB, *sina*, and DG17 proteins. Ten of 24 amino acids between the 4-1BB and *sina* proteins are identical, and 3 of 24, are conservative substitutes. The conserved pattern suggests that these amino acids are functionally important. The *sina* protein is localized in the nucleus, suggesting that it has a regulatory function in cells. The fact that the amino acid sequence of 4-1BB contains features like a zinc finger motif, a nuclear protein, and a receptor domain suggests that 4-1BB may play diverse roles during cellular proliferation and differentiation.

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4-1BB is a 30 kD inducible T-cell antigen, and is expressed predominantly as a 55 K dimer on both $CD4^+$ and $CD8^+$ T lymphocytes. The cytoplasmic tail of 4-1BB contains the sequence, Cys-Arg-Cys-Pro (SEQ ID NO:9), which is similar to the sequence Cys-X-Cys-Pro (SEQ ID NO:10), that mediates the binding of the CD4 and CD8 molecules to $p56^{lck}$ a protein tyrosine kinase[2,3]. An anti-4-1BB monoclonal antibody (53A2 mAb) was used to determine whether 4-1BB may associate with $p56^{lck}$. The 53A2 mAb specifically recognized 4-1BB on a $CD8^+$ T-cell line, CTLL-2, and coimmunoprecipitated a 56 K protein along with 4-1BB. Peptide mapping indicated that the 56 K phosphoprotein was identical to $p56^{lck}$. The comimmunoprecipitation of $p56^{lck}$ with 4-1BB also occurred in nonlymphoid cells such as insect (Sf-21) and HeLa cells when the two recombinant proteins were coexpressed. Analysis of mutant $p56^{lck}$ recombinant proteins showed that two cysteine residues, critical for $p56^{lck}$-CD4 (or CD8) complex formation, are also required for the $p56^{lck}$-4-1BB interaction. These studies establish that 4-1BB physically associates with $p56^{lck}$.

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Figures 31a-c show an analysis of the association of 4-1BB and $p56^{lck}$ in a baculoviral expression system. Figure 31a and 31b show an immunoblot of 4-1BB and $p56^{lck}$. Sf-21 insect cells were infected with 4-1BB-, $p56^{lck}$-expressing recombinant baculoviruses or coinfected with 4-1BB and $p56^{lck}$-expressing recombinant baculoviruses. Total lysates from Sf-21 cells infected with these recombinant baculoviruses were blotted and probed with rabbit anti-4-1BB and rabbit anti-$p56^{lck}$ (Fig. 31a and 31b, respectively). Antigens were visualized with alkaline phosphatase-conjugated secondary antibodies and chromogenic substrates, NBT and BCIP. Anti-4-1BB polyclonal rabbit serum was raised against the oligopeptide, CRPGQELTKQG (SEQ ID NO:13), which corresponds to amino acids 82 to 92 of mature 4-1BB. Figure 31c shows an immune complex kinase assay of $p56^{lck}$. These Sf-21 cell lysates were also incubated with isotype-matched rat IgG_1 (Fig. 31c, lane 1), 53A2 (Fig. 31c, lane 2) or anti-$p56^{lck}$ (Fig. 31c, lane 3). The immune complexes were precipitated, subjected to the in vitro kinase reaction with [γ-^{32}P] and run on a 10% SDS-polyacrylamide gel as described in Fig. 30. The arrow indicates the autophosphorylated $p56^{lck}$ proteins.

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